SOME CYTOLOGICAL ASPECTS OF FERTILIZATION IN THE CROSS BETWEEN THE FUNA (CARASSIUS CARASSIUS, CYPRINIDAE) AND THE LOACH (MISGURNUS ANGUILLICAUDATUS, COBITIDAE)

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Because of their fundamental importance in biology, fertilization phenomena, particularly in crosses between different species, have attracted the attention of many zoologists, from both the cytological and the genetical standpoints. Pioneer contributions with fish in this field were made by Moenkhaus (1904), Morris (1914) and Pinney (1918, 1922, 1928).

Recently Suzuki (1953, 1955, 1957), dealing with embryological and morphological studies of artificial hybridization between the loach (*Misgurnus anguillicaudatus*) and the funa (*Carassius carassius*) or goldfish (*C. auratus*), reported that hybrid eggs showed high mortality at the gastrula and hatching stages of development. The larvae from the funa (♀) × loach (♂) cross survived for 24 days after hatching, while the larvae from loach × funa were extremely abnormal, showing edema. In development the larvae exhibited paternal influences in number of myotomes and melanophores and the size of the nuclei. The evidence presented seems to indicate that the fusion of the egg and the sperm nuclei might have occurred in fertilization. In artificial insemination with the sperm of the loach (*Barbatula toni orcas*) and the egg of the funa (*Carassius auratus*) made by Kobayasi and Yamabayashi (1958), a part of the eggs showed no observable abnormality in the course of their development; the larvae hatched and grew normally, with general external features characteristic of the funa. Quite recently, the present author (Kobayasi, unpublished) undertook a hybridization study between the funa and the loach (*Misgurnus anguillicaudatus*), identical with that of Suzuki (1953, 1955, 1957), and found that a part of the eggs from the cross developed normally, and that the larvae which hatched grew into fishes having the general features of the funa.

The greater part of the earlier work in this field has been morphological, and important cytological events regarding fertilization have remained unexplored. Cytological data are significant and interesting in providing a means of understanding the problems underlying fertilization phenomena. The present author undertook cytological investigations on the hybridization between funa and loach, with special attention to the behavior of spermatozoa during insemination and morphological features of the chromosomes in cleaving eggs.

Materials and Methods

The hybrid combination dealt with in the present study was between the funa (*Carassius carassius*)♀, belonging to the Family Cyprinidae, and the loach

(*Misgurnus anguillicaudatus*)♂, a member of the Family Cobitidae. They were caught in a suburb of Asahigawa during the breeding season. Ripe eggs were obtained from funa females which had received injections of a suspension of loach pituitary bodies, and were placed in vessels containing water regulated at 27° C. Insemination was carried out artificially at temperatures from 26° C. to 28° C. The eggs derived from any single specimen were always divided into two lots, one of which was inseminated with the sperm of the loach, while the other lot was left without insemination for a control. In order to avoid any complications, insemination with the sperm of the same species was not performed in the present experiment. Certain numbers of eggs were taken out at required intervals for fixation. As a fixative, Bouin's solution was employed. Eggs obtained in July, 1960, provided the material for the present studies. Sections were prepared 10 micra thick by the ordinary paraffin method. They were stained with Delafield's hematoxylin and eosin. Smears of spermatozoa were made and stained with gentian violet.

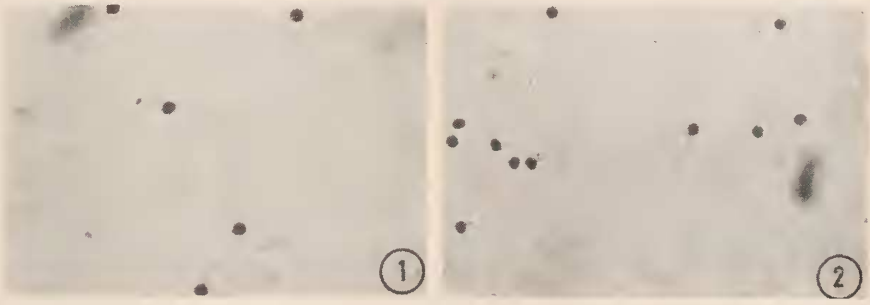

FIGURE 1. Spermatozoa of funa (*Carassius carassius*). × 600.
FIGURE 2. The same of the loach (*Misgurnus anguillicaudatus*). × 600.

RESULTS OF OBSERVATIONS

1. *Spermatozoa of the loach and funa*

Spermatozoa of the loach and funa are similar, each consisting of a head, a middle piece, and a tail without any supplemental filament. The tail appears to attach to the margin of the middle piece.

The spermatozoa of the two kinds of fishes differ from one another in form and size of the head, as well as in size of the tail. The head of the loach sperm is spheroidal, usually 3.0 micra in long axis and 2.8 micra in short axis, with a tail about 18.5 micra long. The head of the funa sperm is spherical, approximately 3.2 micra in diameter, having a tail about 14.5 micra in length (Figs. 1 and 2).

2. *The ovum of funa*

The ripe egg is nearly spherical in form, 1.4 to 1.7 mm. in diameter, with opaque orange coloration. The egg envelope is comparatively thick and tough, consisting of external and internal layers. The external layer is strongly adhesive when the egg is immersed in water. The micropyle, penetrating the enve-

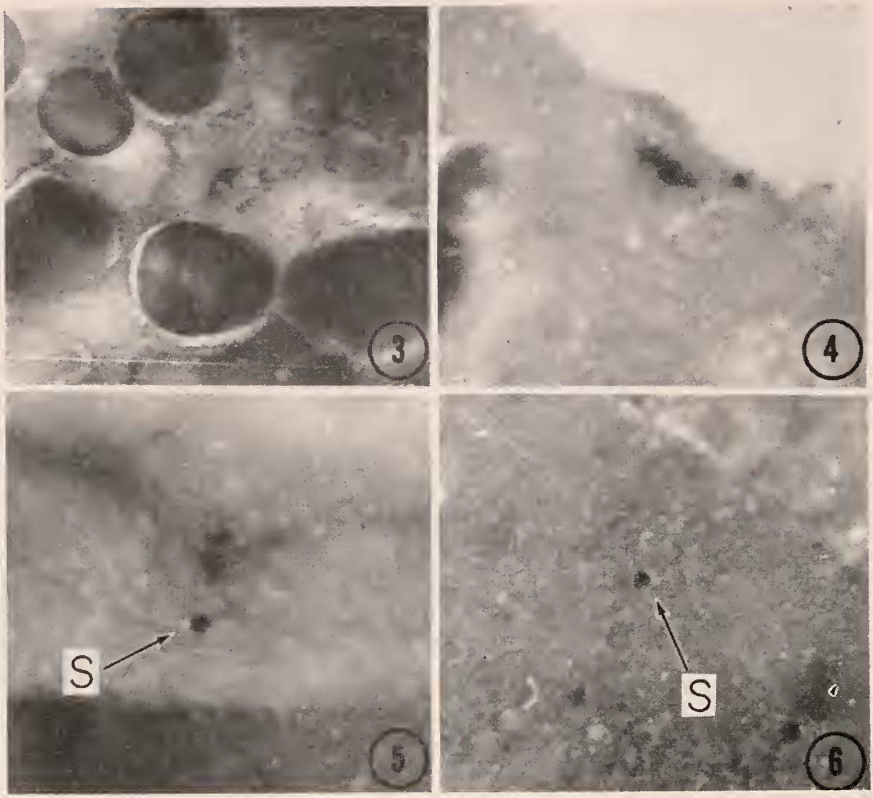

FIGURE 3. Second polar division, metaphase. From an egg of funa just after spawning, polar view. × 800.

FIGURE 4. The same view. × 800.

FIGURE 5. Insemination of loach sperm into funa egg through micropyle, just after insemination. × 800. S, sperm nucleus.

FIGURE 6. A sperm nucleus of the loach in funa egg, three minutes after insemination. × 800. S, sperm nucleus.

lope at the animal pole, is 5.5 to 6.2 micra in upper diameter, and 3.5 to 4 micra in lower diameter. The egg proper lies directly beneath the membrane. The cortical alveoli of various sizes are embedded in the cortical layer of the ooplasm. At the time of ovulation the egg shows the metaphase spindle of the second polar division lying in the periphery of the animal pole, nearly under the micropyle, in a slightly oblique direction (Figs. 3 and 4).

3. *The entry of the spermatozoon of the loach into the egg of funa and the formation of the pronuclei*

Sections of eggs fixed just after insemination showed the entrance of the spermatozoon into the egg through the micropyle (Fig. 5). In many eggs fixed 3 to 5 minutes after insemination, the penetration of the spermatozoon into the egg proper, together with the break-down of cortical alveoli, was observed (Fig. 6).

The sperm-tail could not be detected in the section. It was difficult to recognize clearly the middle piece in the fixed material. After insemination, the second polar division gradually progressed, with separation of the chromosomes at anaphase. With the passage of time, the chromosomes migrated to the opposite poles of the spindle (Fig. 7). The poly- or dispermic condition was not observed. Seven to 10 minutes after insemination, the sperm nucleus turned 180 degrees, and directed its base, with sperm asters, towards the center of the egg (Fig. 8). By this time the polar division was completed at telophase (Fig. 9). Then, the sperm nucleus developed into a vesicular body with the definite appearance of the astral rays, after which was formed the second polocyte. An egg taken at about 12 minutes after insemination, as seen in Figure 10, showed a vesicular sperm nucleus and the second polocyte. Generally, the second polocyte was completed about 15 minutes after the egg was inseminated.

The chromosomes lying in the egg body became indefinite in outline and came together to form a mass of irregular outline (Fig. 11). In the meantime, a membrane appeared, surrounding the mass, although it is not well-defined. Then the nucleus became vesicular by vacuolization. In the meantime, the egg nucleus metamorphosed into a female pronucleus, inside which many weakly stained chromatin elements were scattered (Figs. 12–13). Along with the formation of the female pronucleus, the sperm nucleus completed its metamorphosis into a vesicular male pronucleus. It was nearly spherical in shape and smooth in out-

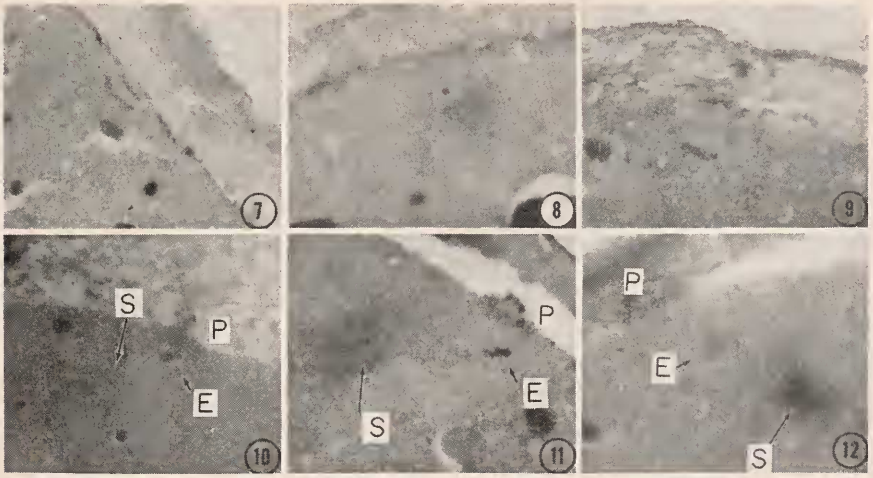

FIGURE 7. Second polar division, anaphase. From funa egg, 5 minutes after insemination. × 240.

FIGURE 8. Sperm nucleus and sperm aster, 7 minutes after insemination. × 240.

FIGURE 9. Second polar division, telophase, 7 minutes after insemination.

FIGURE 10. Extrusion of the second polocyte, sperm nucleus and sperm aster, 12 minutes after insemination. × 240. S, sperm nucleus. P, second polocyte. E, egg nucleus.

FIGURE 11. Second polar body, egg nucleus, and sperm nucleus before metamorphosis, 15 minutes after insemination. × 240. S, sperm nucleus. P, second polocyte. E, egg nucleus.

FIGURE 12. Second polocyte, egg nucleus before metamorphosis, and sperm nucleus in process of metamorphosis, 15 minutes after insemination. × 240. S, sperm nucleus. P, second polocyte. E, egg nucleus.

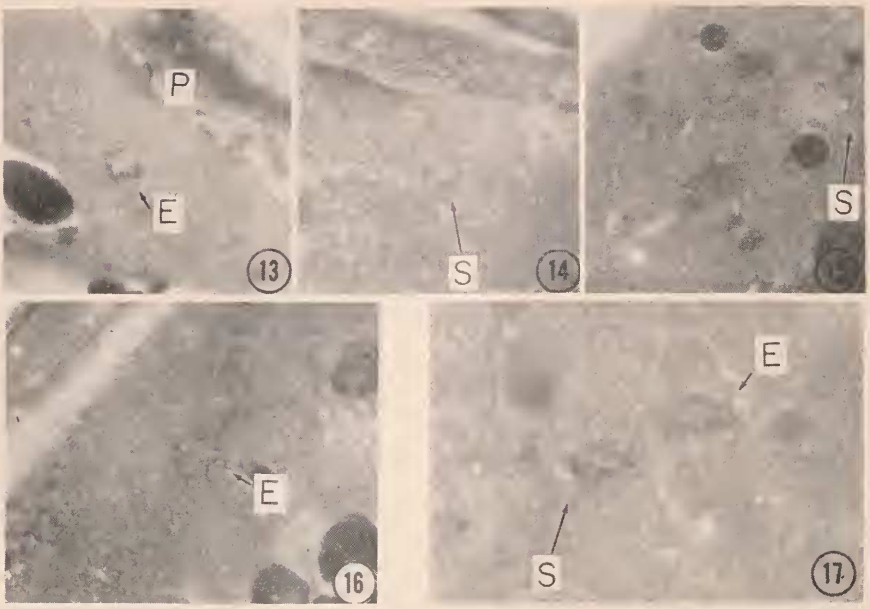

FIGURE 13. Female pronucleus in process of metamorphosis; from an egg, 15 minutes after insemination. × 300. P, second polocyte. E, female pronucleus.

FIGURE 14. Male pronucleus in process of metamorphosis, 15 minutes after insemination. × 300. S, male pronucleus.

FIGURE 15. Male pronucleus after completion, 20 minutes after insemination. × 300. S, male pronucleus.

FIGURE 16. Female pronucleus found in the same egg with Figure 15. × 300. E, female pronucleus.

FIGURE 17. Two pronuclei just prior to conjugation, 20 minutes after insemination. × 600. S, male pronucleus. E, female pronucleus.

line, carrying a fully developed sperm aster with radiation of rays (Fig. 14). It contained many minute chromatin bodies which stained weakly with hematoxylin.

At the time of completion, the female pronucleus lay in the egg periphery, being slightly larger in size than the male pronucleus. Figure 15 shows the latter at the stage of migration in an egg fixed 20 minutes after insemination. The female pronucleus found in the same egg is shown in Figure 16. Figure 17 is a section of an egg obtained 20 minutes after insemination, showing the male and female pronuclei just prior to conjugation. The astral radiation became inconspicuous with the migration of the two pronuclei to the deeper part of the egg.

The course of metamorphosis of the sperm nucleus of the loach in the egg of the funa, as observed in the present study, seems to follow the pattern described by Ojima (1943) in the normally fertilized eggs of the carp, and by Yamamoto (1952) in those of the dog-salmon.

Unfertilized eggs of the funa were immersed in water and observed for control. They were activated in fresh water, and showed the breakdown of cortical alveoli, elevation of the chorion, and extrusion of the second polocyte. The time required for each stage in control eggs appeared to be nearly the same as that in experi-

mental eggs. The unfertilized eggs remained without advancing further in development. A similar situation was reported by Yamamoto (1951) to occur in unfertilized eggs of the dog-salmon.

4. *Migration of the male and female pronuclei*

It is a matter of special note that the migration is more striking in the male pronucleus than in the female pronucleus. The penetration of the spermatozoon occurred always around the top of the animal pole where the micropyle exists. At first the spermatozoon was found lying in the periphery of the egg. About 15 minutes after insemination, or following the extrusion of the second polocyte, a migration of the sperm nucleus took place towards the deeper part of the egg, to conjugate with the egg nucleus.

The second polar spindle was generally formed near the point of sperm penetration. After the extrusion of the second polocyte, the chromosomes left in the egg vacuolized into a vesicular body which was transferred into the female pronucleus. The latter moved slightly towards the center of the egg. About 20 or more minutes after insemination, the two pronuclei conjugated in the place where the female pronucleus already lay. After the conjugation the pronuclei persisted in their places throughout the whole period in preparation for the first cleavage. The pattern of the movement of the female pronucleus as observed in the present material is nearly similar to that reported in the carp eggs by Ojima (1943).

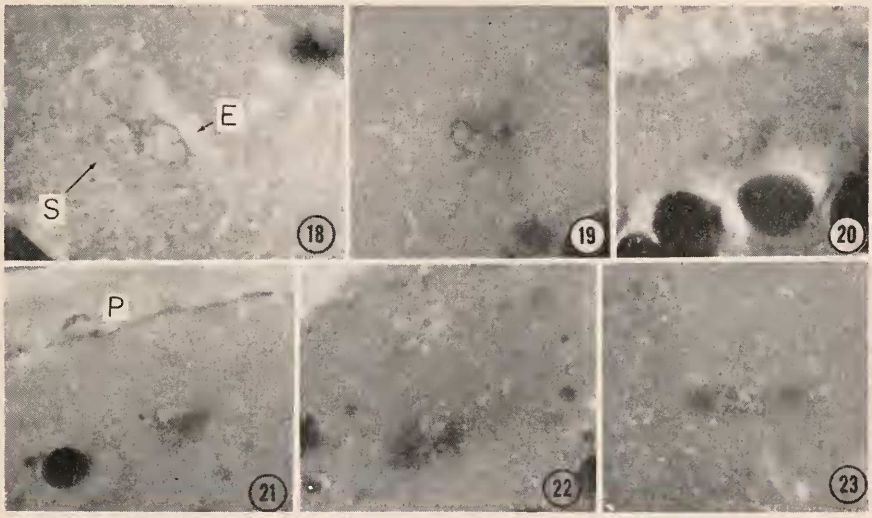

FIGURE 18. Two pronuclei during conjugation, 20 minutes after insemination. × 480. S, male pronucleus. E, female pronucleus.

FIGURES 19–20. Pronuclei after conjugation, 25 minutes after insemination. × 240.

FIGURES 21–22. Two centrosomes, and the first cleavage at prophase. From eggs about 25–30 minutes after insemination. × 240. P, second polocyte.

FIGURE 23. Prophase of the first cleavage. From an egg 30 minutes after insemination. × 240.

5. *Conjugation of the male and female pronuclei*

About 20 minutes after insemination, the male and female pronuclei conjugated in the eggs. The eggs obtained about 25 minutes after insemination showed in most cases the conjugation of the two pronuclei. In Figures 18 to 20, are shown the male and female pronuclei in process of conjugation.

In conjugation, they lie side by side with the nuclear membrane in intimate contact (Figs. 19–20). After conjugation, there is no actual fusion of the two pronuclei at all, but they keep themselves in close contact, distinctly separated by nuclear membranes. Further, this condition persisted unaltered throughout the whole period in preparation for the first cleavage. The staining capacity of the pronuclei increased gradually just after conjugation, but the male and female pronuclei were indistinguishable from each other by their staining capacity or size: they were quite alike in general appearance at the time of conjugation and there was no identifiable character by which they could be distinguished from each other. Sooner or later the astral system developed from the two centrospheres lying on opposite sides of the conjugation plane of the nuclei (Figs. 21–22). The astral system developed radial rays in every direction. At this stage, the chromatin elements in each nucleus became distinct and were well defined by their affinity for stain.

6. *The first cleavage*

Approximately 30 minutes after insemination had taken place, the great majority of the eggs examined were found at prophase or metaphase of the first cleavage division (Figs. 23–24). The eggs preserved about 35 minutes after insemination showed the anaphase or telophase spindle of the first cleavage. It was difficult to distinguish clearly the two different groups of chromosomes, paternal and maternal in their origin, at the stage of metaphase. At anaphase, each chromosome divided into equal halves and the halves migrated to opposite poles (Fig. 25). So far as the present observations went, the migration of daughter

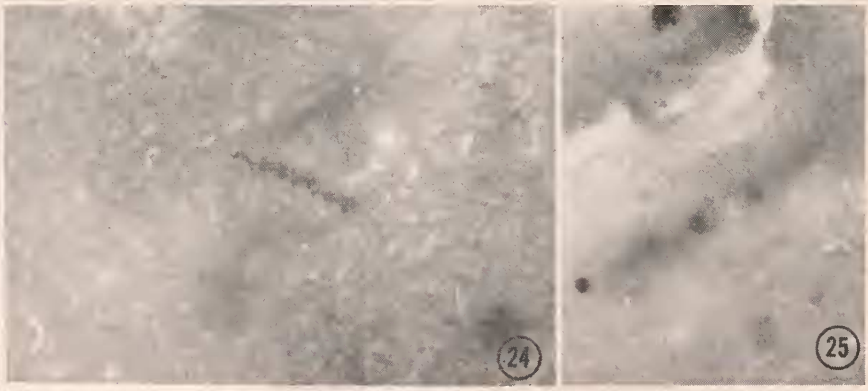

FIGURE 24. Metaphase of the first cleavage. From an egg 35 minutes after insemination. × 600.

FIGURE 25. Anaphase of the first cleavage, 35 minutes after insemination. × 300.

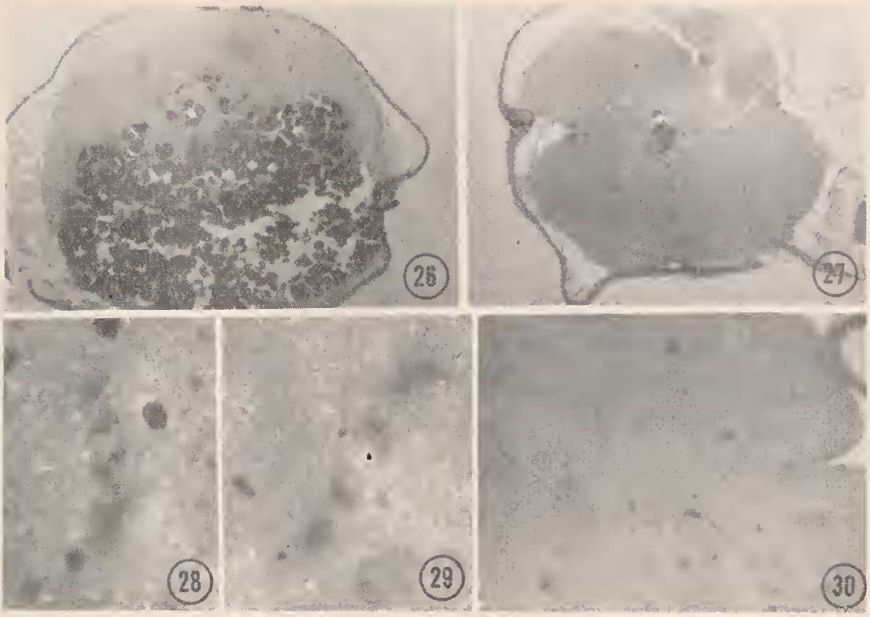

FIGURE 26. Two daughter nuclei after the first cleavage division. From an egg 40 minutes after insemination. × 150.

FIGURES 27–28. Anaphase spindle of the second cleavage. From an egg 60 minutes after insemination. × 300.

FIGURE 29. Anaphase spindles of the third cleavage. From an egg one and a half hours after insemination. × 75.

FIGURE 30. Metaphase of the sixth cleavage. From an egg two and a half hours after insemination. × 150.

chromosomes to the poles was almost synchronous, and abnormal behavior of chromosomes, such as elimination or lag of certain chromosomes, was not observed at all in the course of this division. The reconstruction of two daughter nuclei was completed about 40 minutes after insemination (Fig. 26). It is of interest that the second polocyte was found unaltered in its original position in the egg at the first cleavage.

It was by no means possible to count the actual number of chromosomes at metaphase or any other stages of the first cleavage.

7. *Early cleavages*

Early cleavages took place successively at intervals of about 15 to 20 minutes following the first cleavage. The resting stage in cleavage was short in time. The eggs taken about 50 minutes or more after insemination showed several blastomeres with spindles of various dividing phases. Though the observations were based on six eggs at early cleavage stages, the spindles of dividing blastomeres showed nothing irregular in the behavior of chromosomes; there was neither lag nor elimination of the chromosomes in the material so far observed by the author. Examples are shown in Figures 27 to 30.

Discussion

Moenkhaus (1904) made a cytological study of the process of fertilization in the cross between *Fundulus* and *Menidia*, and reported that about 50% of the eggs from the *Fundulus-Menidia* cross were dispermic, while in the reciprocal cross there were only a few which were poly- or dispermic. Loeb (1912), in discussion of the inheritance of omitted paternal characters in hybrids between *Fundulus* and *Menidia*, and between *Ctenolabrus* and *Stenotomus*, without cytological study of those eggs, stated that the sperm in these cases acted as a parthenogenetic agent. In the present study, artificial insemination of eggs of the funa (Cyprinidae) was undertaken with the sperm of the loach (Cobitidae). The results proved that monospermic insemination occurs in the normal manner, and that the head of the loach sperm metamorphosed into a male pronucleus, followed by conjugation with a female pronucleus of the funa egg. The cleavage of the hybrid eggs was found to proceed regularly. So far as the above features have shown, there is no evidence that, in the cross between funa and loach, the sperm acts as a parthenogenetic agent.

It was shown in the present experiments that after the penetration of the loach sperm into the funa egg, the metamorphosis of the spermatozoon and the egg nucleus into the male and female pronuclei, and the extrusion of the second polocyte in the egg, had taken place in a way comparable to that observed in the normal fertilization of some teleost fishes, for instance the carp (Makino and Ojima, 1943; Ojima, 1943) and the dog-salmon (Yamamoto, 1952). Also, the behavior of the pronuclei, before and after their conjugation in the egg, and the formation of the first cleavage spindle were proved to follow a normal pattern. After conjugation there was no actual fusion between the two pronuclei; a similar feature was reported in the fertilization of the carp (Ojima, 1943), dog-salmon (Yamamoto, 1952) and *Fundulus* × *Ctenolabrus* (Morris, 1914). This is a feature commonly found in other forms of vertebrates (*cf.* Wilson, 1928; Makino, 1934).

In eggs from the cross between *Fundulus* and *Ctenolabrus*, Morris (1914) observed that some chromosomes of male origin lagged at anaphase of the first and later cleavages. In the hybrid eggs from *Fundulus* × *Ctenolabrus*, *Stenotomus* × *Ctenolabrus*, *Fundulus* × *Prionotus* and *Menidia* × *Ctenolabrus*, Pinney (1918, 1922, 1928) announced that lag and elimination of some chromosomes of paternal origin occurred at anaphase of the first and later cleavages. So far as the present study is concerned, there is no evidence for the occurrence of abnormalities such as lag and elimination of the chromosomes. It should be mentioned, however, that the behavior of the chromosomes in the late stages of development remains unknown, due to poor material. Furthermore it was difficult to examine exactly the number of chromosomes in the cleavage spindle. Here, some note may be needed on the chromosomes of the loach and funa. It was reported by Makino (1939, 1941) that the diploid number of chromosomes was 52 in the loach (*Misgurnus anguillicaudatus*) and 94 in funa (*Carassius carassius*). Morphological difference between the chromosomes of the two species was very striking. In parallel to the systematically distant relationship between these two species, the chromosomes differed both numerically and morphologically. It is surprising to see that, between those two distantly related species, insemination and early cleavages took place without visible abnormality.

It was noted in the present material that the second polar body was found lying in its original position in the egg at the first cleavage. This is proof that the second polar body took no part in the formation of the first cleavage spindle.

Suzuki (1953) studied fertilization in the cross between the goldfish and loach and reached the conclusion that the hybrids might be produced as a result of the conjugation of the egg and sperm nuclei. But, there is no cytological proof for this conclusion. The present study seems to be of value in establishing with cytological evidence that, in the cross of funa × loach, insemination actually occurs and early cleavages of fertilized eggs proceed without detectable abnormality.

It is especially interesting to see that the cross between funa and loach yielded hybrid larvae growing in a normal manner (Kobayasi and Yamabayashi, 1958; Kobayasi, unpublished). In hybrid larvae derived from crosses with *Fundulus*, *Ctenolabrus* and some others, Morris (1914) and Pinney (1918, 1922, 1928) reported the appearance of abnormalities in greater or lesser degree. They showed further that there were lag and elimination of some chromosomes of paternal origin at anaphase of the cleavage of the hybrid eggs. The present author found that some hybrid larvae from the cross of funa and loach showed maternal characters very distinctly. However, since no abnormal division was observed in the cleavage of the hybrid eggs, the cause of the maternal effect remains in doubt and a subject for future investigation.

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Summary

1. Insemination and early cleavages were cytologically investigated in the cross between the funa (*Carassius carassius*) and the loach (*Misgurnus anguillicaudatus*), belonging to different families. Eggs of the funa were artificially inseminated with loach sperm at water temperatures from 26° C. to 28° C.

2. Monospermic insemination was cytologically proved; a single spermatozoon of the loach entered the funa egg through the micropyle. Following insemination, the second maturation division of the egg proceeded. About 15 minutes after insemination, the extrusion of the second polocyte occurred. Then the formation of female and male pronuclei followed. About 20 to 25 minutes after insemination, the meeting of the male and female pronuclei took place. In conjugation, both pronuclei came into close contact, side by side, with the nuclear membrane intact, without evidence of actual fusion.

3. It was difficult to distinguish clearly groups of chromosomes of paternal and maternal origin or to count the chromosome number in the first cleavage metaphase. During the period from anaphase to telophase, separation of the chromosomes took place synchronously, without evidence of lag or elimination of chromosomes. The first cleavage was completed 35 to 40 minutes after insemination.

4. Early cleavages took place at intervals of 15 to 20 minutes after insemination. No abnormality was detected in the cleavage spindle. The second polar body was found lying in its original position at the first cleavage.

5. Evidence presented indicates that both insemination and early cleavage followed normal cytological patterns, and consequently that there is no cytological evidence in this cross to show that the sperm acted as a parthenogenetic agent, in spite of the maternal characters noted in larvae resulting from this cross.

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